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STUDIES OF FERTILIZATION IN PLATYNEREIS MEGALOPS

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE OGDEN GRADUATE SCHOOL
OF SCIENCE IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

(DEPARTMENT OF ZOÖLOGY)

APR 6 1917

BY
ERNEST EVERETT JUST

CHICAGO
1915

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BREEDING HABITS OF THE HETERONEREIS FORM OF PLATYNEREIS MEGALOPS AT WOODS HOLE, MASS.

E. E. JUST.

Verrill ('73) first described *Platynereis megalops* figuring in his "Report" a male of the heteronereid phase. Later ('79) he figured the nereis-form and the female of the heteronereis-form changing the name he first gave, *Nectonereis megalops*, to *Nereis megalops*. Andrews, who ('91) had discovered the egg *Nereis limbata*, in a paper on the eyes of annelids speaks of the worm as *Nereis alacris*. I am indebted to Dr. J. Percy Moore who identified the animal as *Platynereis megalops*, Verrill. The belief seems to prevail that in the study of the cell lineage of *Nereis* ('92) Wilson indiscriminately used the males and females of *Nereis* and *Platynereis*. But this belief is by no means founded on any statement in Wilson's paper. Bonnevie ('08) has perhaps strengthened popular misconception through her descriptions of the "two varieties" of *Nereis limbata* at Woods Hole.

I. SWARMING HABITS.

The swarming of *Platynereis* is closely similar to that of *Nereis* (cf. Lillie and Just). There seems to be some variations as noted below. The behavior shows as that of many other forms a definite lunar periodicity: the sea-urchins (Tennent), the Japanese palolo (Izuka), the Pacific palolo (Woodworth and others), the Atlantic palolo (Mayer), *Amphitrite* (Scott), *Nereis dumerilii* (Hempelmann), etc.

Observations were made during the seasons of 1911, 1912 and 1913 at the Marine Biological Laboratory, Woods Hole, Mass. The swarming habits of *Platynereis* have not been worked out as fully as have been those of *Nereis limbata* (Lillie and Just, '13). In the first place during 1911 and 1912 attention was focused mainly on the swarming habits of *Nereis;* moreover, at all times the primary object in the collecting of *Platynereis* was for

experimental study. Strict watch, however, was kept on these worms throughout the summers named. This is especially true for the summer of 1913; during June, July, and August I went out every night, giving attention wholly to *Platynereis* swarming.

The animals on swarming nights swim near the surface of the sea, the males invariably appearing first, the females later. The females rarely exceed fifteen, and indeed on some nights no females swim, while the number of males may be very large. Verrill ('73) says that the worms swim at noon. I have never noted this.[1] Hempelmann ('11) might lead one to think that *Nereis dumerilii* swarms early in the morning. I looked for this during August, 1913, but did not find *Platynereis* swarming before or at sunrise. The evening swarm may last two hours.

The small reddish males swim with great rapidity in an ever more narrowing circle within the patch of light thrown by the observer's lantern until the swarm is at its height. Here and there often at a greater depth than the males swims with slow and even laborious movements, the larger female, pale yellow in color with a thin dorsal line of green—the remnant of the empty gut. One cannot but suspect that the sex ratio in some way depends on the rate of movement: the females are easy prey for fish, the males must easily escape their enemies. The sex ratio of the captured animals must be also influenced by the fact that the females tend to keep further below the surface than the males. This is true of *Autolytus* to a marked degree as I have repeatedly observed. (So too, Andrews, '92, and Mensch.) Verrill, however, says of *Nereis limbata* that in their burrows "there are few males in proportion to the females"—as in the case of *Platynereis*, the reverse is true of these worms during swarming.

As the male comes in the vicinity of female he swims very rapidly in spirals tangential to the surface. They swim together and after copulation and egg-laying, the female slowly sinks from view.

The swarming occurs nightly throughout the months of July and August during the dark of the moon. From new moon to full moon, whether there be moonlight or not the animals do not swarm. Only mature animals swarm.

[1] In July, 1914, I found spent males swimming during the day.

I have never taken this Heteronereid at Woods Hole earlier than June 29. In 1911 I remained at Woods Hole until September 18; I took no worms after August 24. For 1913, August 19 is the date of last capture.

The following tables selected from data of 1911, 1912 and 1913 give some idea of the lunar periodicity of the swarming:

TABLE I. 1911.

(Date of first capture, July 20.)

Moon Phase.	Date.	Number of Females.	Number of Males.
Full moon	August 8	0	0
	9	0	0
	10	0	0
	11	0	0
	12	0	0
	13	0	0
	14	0	0
	15	0	0
	16	1	6
Third Quarter	17	0	0
	18	0	0
	19	0	0
	20	0	0
	21	4	5
	22	6	6
New Moon	23	8	8
	24	10	10
	25	0	0
	26	0	0
	27	0	0
	28	0	0

Comparison with *Nereis* shows in the first place that the number of worms swarming is not so great. It was found, for instance, in collecting *Nereis* to be practically impossible to make an accurate estimate of the number of males; for that reason a record was kept of the females only. On two or three nights only did I find it impossible to estimate the number of *Platynereis* males swarming; on other evenings it was easily possible to count them. The swarm of males on the evening of August 11, 1912, was wonderful. For a few minutes the sea was alive with thousands of the rapidly swimming Heteronereids. In 1913 there was a similar swarm of females, but in no such numbers. As in the case of *Nereis* the collections were made in one place during the three years.

The season, moreover, appears to be shorter than that of

TABLE II. 1912.

Moon Phase.	Date.	Number of Females.	Number of Males.
	June 4 to July 2	None	None
	July 3	0	1
	4	0	0
	5	0	0
	6	0	0
Third Quarter	July 7	0	0
	8	0	0
	9	0	0
	10	1	3
	11	3	3
	12	3	3
	13	0	2
New Moon	14	2	2
	15	0	2
	16	0	1
	17	0	0
	18	0	0
	19	0	0
	20	0	0
First Quarter	21	0	0
	22	0	0
	23	0	0
	24	0	0
	25	2	1
	26	1	0
	27	0	1
Full moon	28	0	0
	29	0	0
	30	15	30
	31	2	30
	August 1	4	30
	2	3	30
	3	0	20
	4	0	0
Third Quarter	5	8	20
	6	14	20
	7	0	0
	8	12	10
	9	10	50
	10	12	100's
	11	15	1000's
New Moon	12	18	30
	13	0	0
	14	0	0
	15	3	6
	16	0	0
	17	0	0
	18	0	0
First Quarter	19	0	0
	20	0	0
	21	0	0
	22	0	0
	23	0	0
	24	0	0
	25	0	0
	26	0	0

TABLE II. 1912.—*Continued.*

Moon Phase.	Date.	Number of Females.	Number of Males.
Full moon	27	0	1
	28	0	1
	29	0	0
	30	0	0
	31	0	0

TABLE III. 1913.

Moon Phase.	Date.	Number of Females.	Number of Males.
	June 13–28	None	None
	29	0	1
	30	0	0
	July 2	0	1
		None	None
	17	0	1
Full moon	18	0	10
	19	0	30
	20	0	20
	21	1	25
	22	0	10
	23	0	8
	24	2	4
	25	50	30
Third Quarter	26	2	4
	27	5	3
	28	16	20
	29	5	8
	30	20	16
	31	21	8
	August 1	6	6
New Moon	2	1	20
	3	4	25
	4	0	16
	5	0	0
	6	0	1
	7	0	0
First Quarter	8	0	0
	9	0	0
	10	0	0
	11	0	0
	12	0	0
	13	0	0
	14	0	0
	15	0	0
Full Moon	16	0	2
	17	1	0
	18	0	1
	19	0	2
	20	0	0
	21	0	0

Nereis. In this my observations approximate those of Verrill. Also, the yearly swarming shows more variations than that of

Nereis. This is strikingly brought out by a study of the tables—especially when one recalls that I gave attention wholly to *Platynereis* for the year 1913. Curves of the runs of *Platynereis* would show that the heights tend to fall in with those of *Nereis*. The lunar periodicity is therefore more like that of *Nereis limbata* than that of *N. dumerilii* which in some respects *Platynereis* resembles.

II. Egg-Laying.

Males and females caught with a hand-net in the evening at the surface of the water and kept in separate dishes may be studied in the laboratory. If a male be transferred with a female to a dish of clean sea-water, the phenomena observed in the sea may be readily followed. The female packed eggs discernible through her pale thin body wall swims slowly in a straight line; or, with head bent at right angles to the body describes a circle of which the head is the center. The male swims in spirals tangential to the surface of the water. Soon his spirals are along the course of the female, her body finally becoming the long axis of his helical body. He entwines the female through this performance and straightens out, thus clutching her in the twist of his body. If this embrace be in the posterior region of the female's body, the male loosens slightly and pulls himself along the female's body. The task appears to be exacting. Often I have observed a rather small male that had worked himself forward after having grasped an unusually large female near the anal segment fall apparently too exhausted to complete the courtship. As the male slips along forward over the female, he lashes his tail back and forth. The female bends her head as if seeking the tail. If the female keep her body in a straight line, the male must move anteriorly until he entwines her body in the pharyngeal region. He now forms a coil around her head of which his tail is the apex. He thrusts his tail down into the coil of his own body and so into the waiting jaws of the female. The female is quiescent throughout. About six seconds after the female has received the anal segment of the male, the animals separate and eggs stream from the posterior segments of the female. The male may be held for a time by the female; if so he swims around, dragging her. I believe that the eggs

escape, not through gonopores or the like, but through lesions of the body wall (cf. Scott.) Eggs escape from three or more posterior segments, occasionally from anterior segments. If escape by way of the posterior segments be experimentally inhibited, or if the female be slightly disturbed, the eggs seem to burst through the body wall at segments more anterior than otherwise. Females killed at the moment of oviposition show tears in the body wall.

After oviposition—and the whole process just described is in general the event of ten seconds—the female sinks to the bottom of the dish, a mere shred. In the laboratory placed in a little water it remains an irritable sticky mass for a time—incapable of exciting fresh males and finally dies, greatly shrivelled and blackened. Often, however, if flooded with fresh sea water it revives, expands to previous size, and swims around actively, almost perfectly transparent. I have kept these spent females alive for several hours. Since there are no sexual segments as in some annelids, but the whole body is little more than a locomotor ovary, it seems safe to assume that this egg-laying marks the end of the worm's existence.

Both animals must be in healthy condition for this behavior. Active males sometimes grasp females which because of rough handling in capturing are doubtless weak and fail to respond. The active males on the other hand are not very hardy: in the laboratory they rarely live twenty-four hours; one experiment made in 1913, failed to show any difference in the vitality of spent and unspent males. Normal females when placed in dishes with males fail to complete the courtship if the vitality of the male as by rough handling be impaired. Males and females may be kept in the same dish until death; if there be no courtship, there is no oviposition. Female *Platyneris* and male *Nereis* show no excitement when in the same dish, so male *Platynereis* and female *Nereis*. The male *Platynereis* ordinarily will embrace only an unspent female *Platynereis*. But on one occasion (July 23, 1913) all (8) males captured in turn and repeatedly embraced a *Nereis virens* eight inches long whose posterior segments had been lost. Once only I saw a male clutch a female which had extruded part of her eggs after a previous courtship.

The animals will go through this courtship when placed in a

very dense suspension of India ink in sea-water; or total darkness. The reaction, therefore, cannot be due to sight. It is more likely due to some chemical emanation from the gravid female only since the spent female is not attractive to the male (Cf. F. R. Lillie on *Nereis*, '12, '13.)

A male *Platynereis* will embrace at least four females. On the evening of August 24, 1911, for instance, I put a male and a female in a dish. They swam around for a time, then the male wrapped himself about the female just back of the head, he let go, uncoiled himself, his tail remaining in the female's mouth. Immediately after release, he was placed with a second female; a minute later he induced oviposition. After intervals of five minutes he embraced a third and fourth female. In all cases the worms shed eggs. The male placed in fresh sea-water with an active female after an hour (11 P.M., about two hours in the laboratory after capture) failed to make a fifth clutch. Other males embraced two females. During 1912 and 1913 these observations were verified.

If after this egg laying behavior, both animals be removed from the dish or if the eggs be pipetted off as laid the eggs develop and normal swimming larvæ much like those of *Nereis limbata* result. If at the moment of her release by the male the female be put in a dish of clean fresh sea-water, eggs will stream out and subsequently develop.

In all these cases sperm are attached to the vitelline membrane within a hull of jelly which has been secreted through the breakdown of the cortical protoplasm of the egg. As in *Nereis* this jelly formation begins at the moment that the sperm touches the membrane. In *Platynereis* it is easily demonstrated that the inseminated eggs have this jelly when laid. Mechanical pressure either by the male, experimentally, or otherwise, as has been repeatedly demonstrated, will not induce oviposition. Mere clutching however recurrent—even by more than one male is not sufficient stimulus for oviposition. The head of the worm may be crushed—eggs will not escape; if she be cut in two, a few eggs escape. Only after thorough drying on filter paper or on sheer dry linen will the eggs burst through the body wall. If the female be finely minced in sea-water practically all the eggs may

be procured. But eggs got in this way do not develop after insemination; they will not fertilize in sea-water. I have sections of uninseminated eggs killed after having remained upwards of two hours in sea-water; the cortical layer and the germinal vesicle are intact. (So *Nereis*.)

Eggs removed from worms after clutching only have the appearance of eggs from unembraced females—no sperm attached, subsequently no trace of development. Sperm are not found on the female's body (*e. g.*, hypodermic impregnation: cf. Whitman, Gardiner, etc.) or near the anus at the time of egg extrusion.

It appears, therefore, that mechanical stimulus is not sufficient to excite oviposition or sperm shedding. The eggs are not laid during or after the embrace nor are sperm shed unless the male's tail has been in the female's jaws. This, then, is a case of copulation followed by internal insemination. And indeed, the very elaborate and precise behavior indicates this. The sperm swallowed by the female inseminate the eggs in the body cavity, oviposition following immediately.

In 1911 gravid females before and after copulation were killed in Meves fluid but proved too refractory for cutting; in 1912, special precautions were taken. The following fixatives were used: Bouin, Gilson, 10 per cent. formalin, and Hennings mixture. With these mixtures the yolk and oil of the eggs are dissolved out, but the chitin of the jaws still makes the procuring of good sections difficult. In 1912 I thought that I had solved the difficulty when after experiments with various agents I procured with KCl, and KCN in sea-water eversion of the pharynx. But in 1913, these methods gave very indifferent results. Dissection of the jaws gave almost negative results. My best sections are those of July, 1912, killed in Gilson, Series A; those of August, 1912, killed in formalin, Series B, and those of 1913 kept in formalin for five months.

Sections of gravid females killed before courtship show no sperm in the body cavity. Sections of gravid females just after copulation show sperm among the antennæ, in the mouth, in the pharynx, and in the body cavity. The sperm may be traced, therefore, entering the mouth, passing down the pharynx whence they escape through lesions in the pharyngeal wall to the cœlom.

They may be found also attached to the vitelline membrane of the eggs. If one minces a male, one procures not only sperm but large numbers of corpuscles. Apparently, these are not injected into the female's body (cf. Scott on *Amphitrite*).

Since the mechanical pressure of the male, though often repeated, is not sufficient stimulus for egg-laying, it may be assumed that either the sperm or some secretion with, or of them stimulates in the female movements which bring about oviposition. In some cases males after having induced oviposition in two or three females cause egg-laying in a third or fourth as noted above. A slight amount of this substance, therefore if such there be in addition to the sperm themselves, is sufficient to initiate egg-laying. The injected substance, on the same ground could scarcely exert sufficient pressure to stimulate oviposition.

I had projected for 1913 various experiments to determine this point. The first experiment on the list, however, was clear enough to warrant abandoning the others. I put a female in a dish with no water. If a drop of sea-water be put on her head there is no response. Only complete drying causes breakdown of body wall. If instead of pure sea-water the minced female be added there is no response. But if a drop of minced male be added oviposition follows. This observation was made several times.

The following protocol from notes of the night of July 25, 1913, is typical:

Experiment.—Six males cut up in water adherent to their bodies (*i. e.*, not dried). Dried female put in this sperm suspension. No oviposition. A second female placed in the sperm suspension; and a third. No oviposition.

2. Three males cut up in three drops of sea-water. Two successive females used. No oviposition.

3. Six dried males cut up. Two dried females placed with heads in the sperm suspension. Eggs laid. Next day: trochophores.

4. Three males cut up in two drops of sea-water. Two dried females placed with heads in the sperm suspension. (Both females later copulated with males and laid eggs.) No eggs laid.

Oviposition, then, is clearly brought on through the ingesting of sperm with very little sea-water.

Nereis diversicolor O. F. Muller gives birth to living young. *Autolytus* (Agassiz) carries its larvæ in a brood pouch. In both of these forms there is probably internal insemination. Eisig

has described copulation in an annelid, *Capitella*. *Platynereis* is of interest in that oviposition so quickly follows copulation.

III. The Nereis Form of Platynereis Megalops.

As in the case of *Nereis* an attempt has been made to rear the larvæ of *Platynereis*. Best results were obtained during 1913. A table was kept of the development of the young worms and their characteristics noted. They closely resemble the larvæ of *Nereis dumerilii* described by Hempelmann which he obtained from his cultures. Some of my worms aged six months measured four centimeters. It is hoped that a study of these forms will give a clue to the swarming habit.

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THE MORPHOLOGY OF NORMAL FERTILIZATION IN PLATYNEREIS MEGALOPS

E. E. JUST

THREE PLATES (THIRTY FIGURES)

1. INTRODUCTION

In a previous paper on Platynereis megalops, which described the egg-laying habits, it was stated that insemination takes place in the body cavity of the female and, further, that the eggs will not fertilize when inseminated in sea water. The present paper is a description of the normal fertilization process in Platynereis. An experimental analysis of fertilization in Platynereis appears elsewhere (Just, '15).

2. NORMAL FERTILIZATION OF PLATYNEREIS

The living egg. The egg of Platynereis is compressed and irregular in shape while in the body cavity. Those eggs which happen to be uninseminated when laid gradually round out in sea-water as almost perfect spheres equatorially, but with a rather shorter polar axis. The large, centrally placed, germinal vesicle is slightly elongated in the polar direction. The largest eggs, fully rounded out, measure 180 to 200 μ. They are almost perfectly transparent, have an equatorial ring of oil drops, and a well marked transparent exoplasm or cortical layer of protoplasm with very faint granules forming a delicate mesh. In short, the living egg closely resembles that of Nereis; it is larger (but *cf.* Wilson, '92) not so deeply pigmented, and lacks the characteristic yolk spheres of the Nereis egg.

A. Fertilization in the living egg

We may consider the fertilization of the egg under the following heads: (1) insemination, (2) penetration of the sperm, and (3) copulation of the germ nuclei.

1. Insemination. In Platynereis insemination normally takes place in the body cavity (Just, '14). The eggs, when laid, have the sperm attached within a thin hull of jelly, the secretion of the cortical layer. If the worms be allowed to deposit eggs in India ink ground up in sea-water it can be proved satisfactorily that a hull of jelly, as in Nereis, envelops inseminated eggs. This jelly, absent in uninseminated eggs, is formed from the exoplasm of the egg as the result of stimulation through sperm attachment. In sea-water the zone between India ink particles and the vitelline membrane gradually widens, not so much because of the slow diffusion of the jelly from the egg, as because of the swelling of the extruded jelly.

Insemination in some way brings about oviposition. The presence of the sperm in the female is a stimulus to egg laying; as in Nereis (see Lillie and Just) the presence of the sperm in the sea-water brings about the shedding of the eggs. The first result of the attachment of the sperm to the egg is jelly formation through cortical secretion, with the consequent formation of the perivitelline space; and this process must begin in the body cavity, since eggs have a thin jelly investment when laid. As in Nereis the vitelline membrane is preformed; the sperm does not cause 'membrane formation.'

For twenty to thirty minutes after oviposition, the sperm remains external to the egg. During this time profound changes take place in the egg, many of which doubtless are to be interpreted as changes incident to maturation, the mechanism of which is released with the breakdown of the cortical substance and the consequent formation of the perivitelline space. These changes: breakdown of the germinal vesicle, formation of the spindle, polar body formation, and cytoplasmic movements, are easily followed in the living egg.

2. Penetration. In Nereis a striking phenomenon of sperm attachment is the fertilization cone (Lillie, '11, '12). In Platynereis no sharply defined cone is found. There are, however, cytoplasmic disturbances at the point of sperm entry. In polyspermic eggs the cytoplasm may form a low blunt protrusion, with as many as five spermatozoa attached to it, but this is not

a cone. Twenty-five minutes after oviposition a slender strand of protoplasm may be discerned across the perivitelline space and beneath the point of sperm entry; even this does not seem to be constant, but appears to be formed only in the animal hemisphere. This protoplasmic strand lies, first, in a radius of the egg, but gradually bends so that it now lies almost tangential to the egg. It is found after sperm entry.

After thirty minutes the spermatozoon is engulfed. Often the formation of the sperm aster is discernible, the middle-piece and tail remaining outside. The maturation asters are always visible in the living egg. Mathews ('06) has called attention to the difference between the structure of the asters of living eggs and of fixed material. The difference is certainly striking in Platynereis. Instead of the short stiff astral fibres of chrom-osmic material or the long slender ones of mercuric fixation, one sees in the living egg beautiful broad rays sweeping through the cytoplasm.

3. Copulation of the germ nuclei. About fifty minutes after egg-laying, the germ nuclei copulate, the cleavage asters form, and at sixty minutes the egg divides unequally. The egg at this time exhibits a stratification of protoplasmic stuffs. During maturation the cytoplasmic currents shift the materials. The equatorially placed oil drops, about eighteen in number, gradually become massed at the vegetative pole, the coarser (yolk) granules lie above these; at the clearer animal pole are the male and female pronuclei. Beneath the polar bodies the cytoplasm is most transparent. The asters are very distinct. One cannot get an adequate picture of these structures from sections. In the living egg they are incomparably clear; large broad rays which bear little resemblance to the short stiff fibres seen in the sections.

The penetration path of the spermatozoon may often be followed, the copulation path always followed. The spermatozoon enters at any point of the egg and through this, as in Nereis (Just, '12), the first cleavage plane passes along the copulation path of the germ nuclei.

B. Observations on the sectioned egg

Observations of the phenomena of fertilization in the living egg were supplemented with a study of sectioned material.

Technique. Eggs were fixed in Meves' fluid for thirty minutes, one hour, or twelve hours. Aceto-osmic-bichromate mixtures (Mathews,[1] '99; Bensley); Bouin's fluid, modified by the addition of an equal volume of water; and Gilson's mercuric-nitric mixture were likewise used. Although very destructive to the yolk and oil, the modified Bouin proved helpful in the study of certain details in connection with sperm penetration.

The difficulties of fixation, which are great in this egg, as in Nereis, may in large measure be overcome by the subsequent treatment. The following methods were used after fixation with Meves:

(a) Clearing with double distilled anilin oil from 80 per cent alcohol.

(b) Clearing in cedar oil from 95 per cent alcohol.

(c) Clearing in cedar oil from 95 per cent alcohol after treatment with glycerine (eggs put in 70 per cent alcohol plus an equal amount of glycerine).

(d) Clearing in xylol from 95 per cent alcohol or from absolute alcohol.

In all cases xylol was used before imbedding in paraffin or in paraffin with some admixture of Johnston's rubber-asphalt mass. It was found that avoidance of absolute alcohol left the eggs less brittle and therefore less refractory in cutting. By far the most natural contours of both the Platynereis and the Nereis eggs are preserved through the use of aniline oil after 80 per cent—a clearing agent that I have used successfully for several years. Staining was with iron hematoxylin alone. Sections were cut four micra thick.

Spermatozoa, after fixation, were studied for the most part unstained after the methods of Koltzoff, de Meyer, etc. The iodine mixture recommended by Mayer for Volvox proved in-

[1] From the legend of Mathews' figures it appears that he used aceto-osmic-bichromate mixtures.

valuable. For permanent preparations Bensley's staining mixtures were used.

1. Stages previous to the penetration of the sperm. The egg of Platynereis rivals in structure the beauty of the Nereis egg. A section of an uninseminated egg (fig. 1) teased out of the female directly into Meves' fluid gives many of the details. The cytoplasm is sharply marked off into two regions: the exoplasm made up of clear cortex and zone of oil and yolk and the deeply staining endoplasm.

The outer portion of the exoplasm is a mesh of pale blue delicate fibrils, the alveoli of the cortical jelly. The outer limits of this cortical layer—slightly more dense than the deeper portions—is studded with black granules immediately below the vitelline membrane. The inner border of the cortex arises from a zone of closely-packed, deep-staining bodies, from which apparently the walls of the cortical alveoli project. Below this inner border is the region of oil drops which lies in the equatorial zone, among spherules which prove, from their later behavior, to be yolk spheres, although even in the best preparations, the fine granules of which they are composed tend to shrink from their spherical walls (*cf.* Lillie, '11; figure of Nereis egg fixed in Fleming). These yolk spheres are evenly crowded against the deeply stained basal area of the cortex. Around the germinal vesicle and closely applied to it is the endoplasmic mass, made up of fine granules which take the stain very tenaciously. Its outer limits are uneven, encroaching on the area of oil drops and yolk spheres as blunt projections.

Scattered throughout the germinal vesicle, as in Nereis, are the chromosomes—fourteen tetrads. These lie among many black granules of varying size. Although an attempt has been made to study their number, distribution, etc., and to ascertain any constant characters, nothing now can be said further of them. These granules tend to be spherical and to grade down to minute bodies.

The whole egg, therefore, exhibits a granular structure, both living and fixed, as Mathews some time since ('06) for echinoderm eggs and more recently Kite for some other eggs have shown.

Lillie ('06), too, in a most elaborate study on the egg of Chaetopterus, has determined the granular structure of the cytoplasm. Vacuoles found in mercuric-nitric or picro-acetic preparations are filled with yolk or oil in Meves' preparations or in the living Platynereis egg (*cf.* Wilson, '98, on the cytoplasmic structure of eggs, including that of Nereis).

The egg of Platynereis, as compared with that of Nereis fixed with the same methods, does not show so clearly the radial striation in the cortical layer or the homogeneous yolk spheres.

Ten minutes after laying the germinal vesicle is breaking down and maturation asters, formed outside its wall, are pushing into its substance. The deepest of the cortical alveoli are often still unemptied; the whole process of jelly extrusion can easily be followed from its beginning in inseminated eggs. On one or between two of the apices of the wavy vitelline membrane the spermatozoon is found attached by its perforatorium. Sperm head, middle-piece, and tail are readily distinguished (fig. 2).

Fifteen minutes after laying, the cortical jelly has been wholly extruded (fig. 3) and the first maturation spindle formed, with the chromosomes in late prophase. The endoplasm, with the extra-chromatin substance of the germinal vesicle, imbeds the spindle. *In toto* mounts of the egg at this stage, as is true of the Nereis egg, give no view of the spindle. One sees only a deeply stained core of substance which incloses the spindle. The egg is irregular in shape and the vitelline membrane is closely applied.

The spermatozoon is visible on the membrane (figs. 3 and 4) above a group of granules similar to those more thinly scattered throughout the periphery of the egg. These granules are markedly like those described by Meves and are doubtless 'mitochondria;' but in Platynereis they cannot possibly have the significance that Meves ascribes to them in the eggs of various forms. The granules appear massed beneath the point of sperm entry, but these masses assume no definite form. I have purposely figured those that give the nearest approach to cone formation (figs. 3, 4, and 5). A slender strand of cytoplasm may extend toward the membrane just below the perforatorium.

The granules in the region may appear as a disc, but never as a retracted cone, as in Nereis. The cortical breakdown has released the close application of the yolk spheres to the inner cortical margin; they are now irregularly spaced and among them lies the granular cytoplasm.

The figures (2 to 5) also give good pictures of the spermatozoa. They are much like the living spermatozoon. The head is almost spherical, the perforatorium a large blunt cap; the middle-piece and tail are often clearly defined.

2. Penetration of the spermatozoon. The penetration of the sperm head begins at twenty to twenty-six minutes after laying (*cf.* Nereis, forty-five minutes after insemination). The first maturation spindle, in the metaphase, is oriented in the polar plane of the egg; the inner endoplasmic mass which incloses the spindle is, at this stage, triangular in section; the outer aster of the spindle is near the apex of the triangle. The base of the triangle is less blunt than in previous stages and reaches farther outward along a radius of the egg. The various stages of penetration are shown in figures 6 to 18. The sperm substance enters the egg as a slender black thread, which gradually increases in size at its inner end. The sperm head, in my preparations, is usually homogeneously black, but often the external bulb is not so dark; or lighter areas appear along the entering thread; (particularly figs. 10, 11, 12, and 14). Often, especially in sections stained for twelve hours only, in stages just after the attachment of the perforatorium to the cytoplasm, the head appears, not as a homogeneous chromatin mass, but as a slightly differentiated body. One gains, therefore, the impression that the spermatozoon *flows* into the egg (*cf.* Koltzoff and Lillie, who, with different methods, find Nereid spermatozoa extremely ductile).

Cytoplasmic changes due to sperm entry are clearly marked during the later stages of penetration; striae appear in the cytoplasm around the entering spermatozoon, the area stains more deeply, and a projection from the endoplasmic mass reaches out toward the point of sperm entry (see figs. 14 to 17) (*cf.* on these points, Foot, Gardiner, Vedjovsky, Jenkinson, and Lillie, '12).

As the head is drawn into the egg, the inner bulb turns with its growth. Finally, the portion forming the external bulb is engulfed. The middle piece and tail, as in Nereis, never enter the egg (fig. 17). They may often be found in sections outside the membrane after penetration of the sperm head (see figures).

I have never found the spermatozoon in the Nereis or in the Platynereis egg at the time or in the form figured by Wilson ('96 and '00).

Does the sperm head rotate? I could not positively determine the rotation of the sperm head in the egg of Platynereis. In the first place, a definite cone organ, like that of Nereis, is lacking, and secondly, the middle-piece does not enter the egg. The history of the sperm penetration is known practically for every minute from entrance to pronuclear copulation. Meves' fixation alone was not depended upon. The Bouin preparations gave results much like those of Bonnevie's with picro-acetic mixtures on the Nereis eggs. While absolutely worthless for cytoplasmic detail, they were helpful in determining the structure of the sperm nucleus after penetration. The evidence favors rotation; the turning of the inner sperm bulb (fig. 17) and the position of the long axis of the sperm and aster as often found at right angles to the radius of the egg (fig. 22).

The sperm aster does not arise until the nucleus is beyond the yolk region (figs. 14 to 22). Within the endoplasm, the aster once formed, quickly divides equally, but the amphiaster does not long retain its equal poles, for one sperm centrosome and its aster gradually dwindle in size. Rays arise between one or both of the sperm centrosomes and the inner centrosome of the maturation spindle, thus forming a secondary spindle. The sperm nucleus lies nearer the larger sperm centrosome (see figs. 20 to 25).

3. Copulation of the germ nuclei. The egg chromosomes, after the formation of the second polar body, form fourteen chromosome vesicles which fuse to establish the egg nucleus (figs. 26, 27), all vestiges of the egg aster disappearing. The sperm nucleus enlarges as its asters become smaller. At the time of apposition, but one sperm aster is found (fig. 28). I believe that one sperm

aster begins to wane soon after the formation of the homodynamic amphiaster and finally disappears. One aster can always be found (fig. 29). The opposing nuclear membranes break down and one nucleus forms with the single sperm aster. Soon a small aster appears on the nuclear membrane (fig. 30), the nucleus breaks down, and the heterodynamic first cleavage spindle forms.

3. DISCUSSION

The case of Nereis and Platynereis, with respect to the entrance cone offers an interesting parallel with that of Toxopneustes and Arbacia (Wilson and Mathews). In both Nereis and Platynereis, however, the middle-piece is left outside the egg. The absence of cone-organ in Platynereis makes the question of rotation obscure, whereas in Nereis the evidence is indisputable.

Bonnevie ('08), in her paper on Nereis, has mentioned certain cytological differences between the "large and small varieties" of Nereis eggs. As indicated above, the time of sperm entry is earlier in Platynereis. It is also true that the polar bodies are formed earlier, the first cleavage is earlier, and the subsequent rhythms are faster, so that the larval stage is reached earlier.

So far as both Nereis and Platynereis are concerned, the rôle of the middle-piece or its contained centrosome, as the chief actor in fertilization, is wanting. There are spermatocytes with intra-nuclear centrosomes[2] (see Julin on Styleopsis). But if this hypothesis be postulated (*cf.* Packard in 1914) for Nereis sperm, this next step, as was pointed out by Lillie in 1912, should also be taken: the centrosome gradient must be quantitatively different from its base at the middle-piece to the tip of the sperm head: "If intra-nuclear centrosomes are the causes of the formation of the sperm aster, not only must they exist at every level, but also (that) they must decrease in size from the base to the apex of the sperm nucleus!"

[2] See also Hegner and Newman for intra-nuclear centrosomes in oocytes.

According to Schaxel, the middle-piece does not enter the egg of echinoderms. Meves will not admit this for echinids and doubts that in Nereis the middle-piece is left outside the egg while denying the centrosome the chief part in fertilization. In Platynereis, as in Nereis, by diverse methods it can be shown that the middle-piece does not enter the egg. We are thus forced to conclude that, whatever its rôle, the middle piece in Platynereis can play no part, either in heredity or through a centrosome in the dynamics of fertilization.

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DESCRIPTION

All figures were drawn with the camera lucida with Leitz $\frac{1}{12}$ oil immersion objective and No. 5 ocular, except where otherwise stated. All figures from sections of inseminated eggs of Platynereis megalops. All sections from eggs killed in Meves' fluid and stained in iron haematoxylin.

PLATE 1.

EXPLANATION OF FIGURES

1. Section of an unfertilized ovocyte. The oil drops are a delicate brown, the granular yolk spheres very lightly stained. The cortex is intact.

2. Ten minutes after laying. The cortex is partially reduced. The head, middle-piece, and tail are clearly shown.

3 to 5. Fifteen minutes after laying. The granules are massed below the point of sperm attachment; the perforatorium is still attached to the membrane

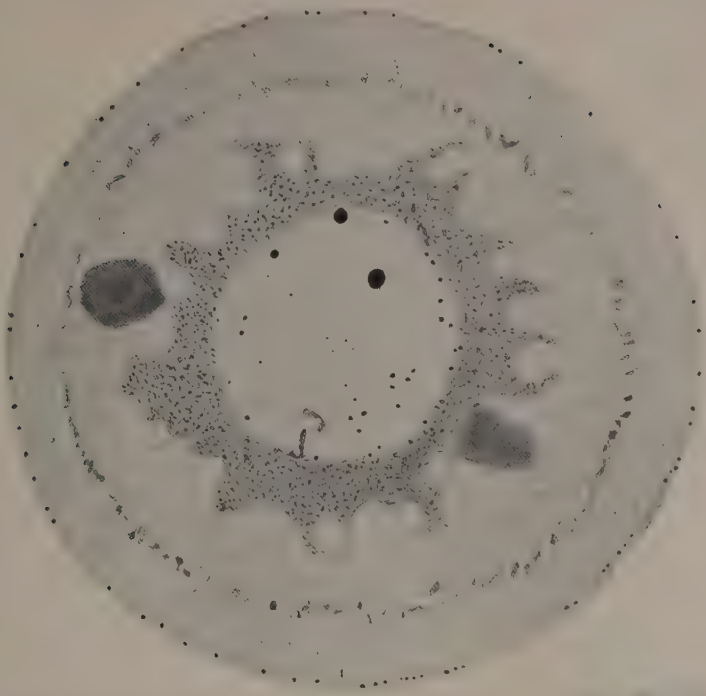
6 to 8. Twenty minutes after laying.

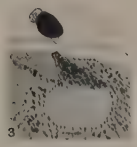
6. The perforatorium is touching the cytoplasm. The granular mass has disappeared.

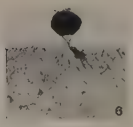
7. The perforatorium is in the cytoplasm.

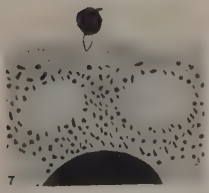
8. A somewhat tangential section, showing the very beginning of penetration.

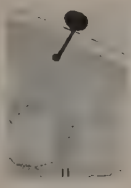
9 to 13. The penetration stages, twenty-five minutes after laying, mesophase of the first maturation division. The figures show that there is no constant disposition of granules at the point of sperm entry—certainly nothing of the nature of a cone, as in Nereis.











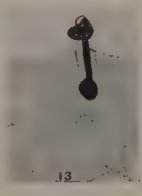


PLATE 2

EXPLANATION OF FIGURES

14, 15. Penetration stages, twenty-five minutes after laying. Note the turning of the inner sperm bulb.

16 to 18. Twenty-seven minutes after laying. The middle piece is shown in fig. 17. In 16 and 18 the middle-piece was found in adjacent sections.

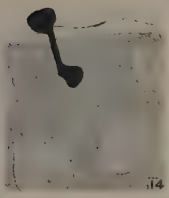
19 and 20. Thirty minutes after laying, telophase, first maturation division. The sperm head is still within the zone of oil drops and without an aster.

21 to 22. Thirty-two minutes after laying; early prophase, second maturation division.

21. The sperm head is at right angles to a radius of the egg, the aster forms around the granule at the tip of the sperm head.

22. Formation of sperm aster within the endoplasm.

23. Thirty-five minutes after laying. The amphiaster is in contact with the egg aster. The spermatozoon is in an adjacent section.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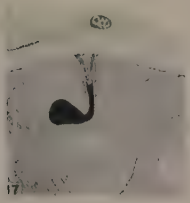
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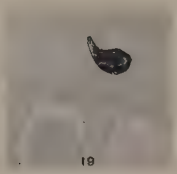
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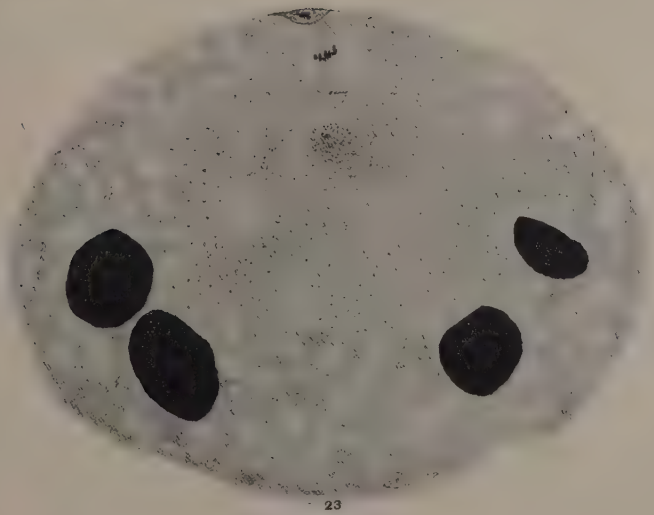
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PLATE 3

EXPLANATION OF FIGURES

24 to 30; oc. 1, $\frac{1}{12}$ oil im. Later stages, showing marked inequality of sperm asters. Note relation of the spermatozoon to the larger aster.

25a and b. Forty minutes after laying.

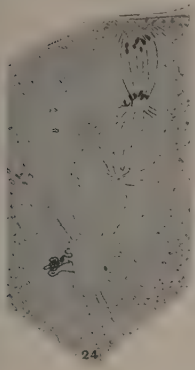
26. Forty-six minutes after laying. The egg aster is degenerating.

27 to 29. Copulation stages.

27. The smaller sperm aster could not be found. Two egg and three sperm nuclear vesicles are shown.

28 to 29. Formation of the male and female nuclei.

30. Origin of the first cleavage spindle.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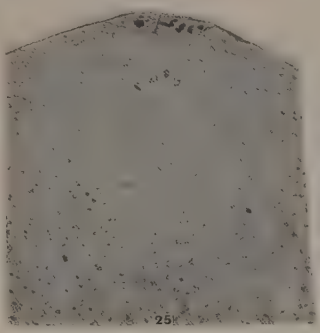
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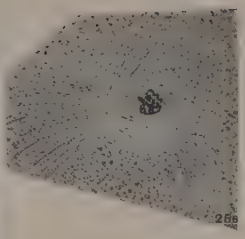
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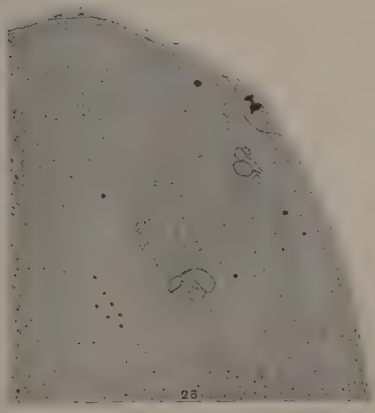
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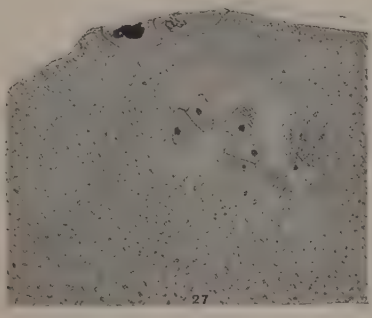
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AN EXPERIMENTAL ANALYSIS OF FERTILIZATION IN PLATYNEREIS MEGALOPS.

E. E. JUST.

Study of the breeding habits of *Platynereis megalops* revealed the fact, as has been pointed out (Just, '14), that insemination takes place in the body cavity of the female and that although egg laying begins often but five seconds after copulation, the eggs will not fertilize when artificially inseminated after exposure to the action of sea-water. It is this failure of sea-water insemination that forms the basis of the present contribution to the analysis of fertilization in *Platynereis*. In order clearly to interpret the phenomena of sea-water insemination a study of the morphology of the normal fertilization was made (see Just, '15*a*).

The experiments undertaken for the analysis of fertilization in *Platynereis* come under three heads:

A. Conditions of successful insemination.

B. Cross fertilization with *Nereis*.

C. Artificial parthenogenesis with various agents.

B and C are taken up mainly because they supplement results under A.

A. Conditions of Successful Insemination.

During the summer of 1911, I was studying the maturation and fertilization of the *Platynereis* egg for comparison with those processes in *Nereis*. The methods of insemination used with *Nereis*, cutting out the eggs and sperm in sea-water, gave no cleavage. Various trials with the utmost care, using diverse methods never gave cleavage. Not until August 24, 1911, did I chance to find that normally insemination takes place in the body cavity of the female (cf. Just, '14).

1. *Observations on Eggs Inseminated in Sea-water.*

If eggs and sperm be cut out of *Platynereis* and mixed in sea-water, the phenomena of maturation, sperm attachment, and

copulation of the germ nuclei may be readily followed; but such eggs do not segment nor do they ever develop into swimming forms.

The Living Egg.

If insemination be made in a suspension of India ink ground up in sea-water, the jelly formation may be easily followed: it differs but little from the cortical outflow observed in eggs normally laid. All eggs, however, do not secrete this jelly; of these, some remain in the germinal vesicle stage and others go through maturation with all or part of the cortex intact.

As in the normally inseminated egg (see Just, '15*a*) no cone is present. More often than in the normally laid egg a broad plateau of cytoplasm marks the point of sperm attachment. The sperm, from one to six, are attached to the membrane above this raised cytoplasm or near it.

Maturation proceeds about as in the normal egg. At maturation stages slightly later than in the normal egg, the sperm may be found in the egg. It moves forward with aster formation. The pronuclei meet, remain apposed for a short time, separate, and fade from view. This is not true of all eggs; for apparently, those in the germinal vesicle stage or in maturation stages with cortex intact never engulf the sperm. Moreover, in many eggs that are in maturation with the cortical layer gone, one cannot find sperm.

These eggs never divide. At first, 1911, I thought that this behavior of the egg was due to injury of the worms. Its significance became clear only after the discovery of the normal method of egg-laying.

The Sectioned Egg.

During four seasons eggs have been preserved at three and five minute intervals upward to two hours after insemination in sea-water. Study of the sectioned eggs confirms the findings of the study of living eggs. Many eggs remain ovocytes with sperm attached or not. Those that go through maturation do so with or without jelly formation. Eggs that form jelly are likewise of two classes: those in which sperm are found to have penetrated and those in which no sperm are found.

I have not been able so far to determine any structural differences in the ovocytes with and without sperm attached. In the case of the eggs that maturate with the cortex wholly or partially intact, the spindle may be abnormal. In most cases if it reach the periphery of the egg it does so at a point practically devoid of cortical cytoplasm. Or again, it may lie parallel to a tangent of the egg membrane.

Those sections which reveal the sperm within the egg are in the minority. It appears from experiments several times repeated during the four seasons of study that the penetration of the sperm depends upon the amount of sea-water used. If the eggs be inseminated in a large quantity of sea-water or washed (by changing the water several times) very few eggs form jelly. With less water more form jelly. Eggs inseminated quickly in small quantities of sea-water are capable of engulfing sperm.

The history of the penetration as known may be briefly given. One finds sperm external to the egg at different stages. How it gets into the egg I cannot yet state with certainty although this point has received most careful study for three years. Material has been prepared in every way possible to demonstrate the early penetration. So far I have not found the sperm entering the egg as a slender thread like that in the normal egg. It can be easily demonstrated in the endoplasm. On one slide of the 1911 series, for instance, I counted twenty sperm heads with their asters lying near the centre of the egg. The sperm head remains for a longer time than in the normal egg a black knot with a long drawn out thread extending to the single aster. A second aster has never been found. The germ nuclei copulate but the eggs never cleave. Various stages are found from sixty to one hundred twenty minutes after insemination—sixty minutes after cleavage in the normal egg. The pronuclei after apposition gradually separate and degenerate as discrete nuclear masses. Many eggs show only one chromatin mass in process of degeneration; doubtless, these are eggs which sperm do not enter. The sections of such eggs closely resemble those of *Nereis* eggs from which the sperm have been removed (see Lillie, '12). I have repeatedly made observations on living eggs inseminated in sea-water and on sections. I have yet to find a single cleaving egg.

Two hours after insemination the eggs exhibit cytoplasmic stratification; the oil drops later fuse to form one at the vegetative pole. Twelve hours after insemination the conditions are the same; there is never a swimming form among these eggs.

2. *Nature of the Inhibition to Development.*

It may be very clearly shown that sea-water is responsible for the lack of cleavage by the method of "dry insemination." If males and females dried on filter paper be cut up separately and the drops of eggs and sperm thus obtained be mixed with subsequent addition of sea-water, a percentage of the eggs always cleave and develop into normal trochophores. I have kept larvae from such dry inseminations until they were seven mm. long with thirty or more segments, few differing from normally laid eggs. There is doubtless an optimum time after mixing for the addition of sea-water, but any time upward to two minutes gives results. The following is an example:

August 3, 1912. To determine the time interval after mixing dry eggs and sperm before adding sea-water.

Water Added.	Per Cent. of Cleavage.
1. At once	60
2. Five seconds after	50
3. Ten seconds after	90
4. Twenty seconds after	45

Practically, as soon as eggs and sperm are mixed, sea-water may be added. I have not been able to add sea-water quickly enough after mixing to prohibit cleavage. If the eggs are allowed to stand two minutes the majority are plasmolyzed by the addition of sea-water.

The amount of sea-water that will permit fertilization has been repeatedly determined:

July 28, 1912, 9:45 P.M. Experiment to determine the maximum amount of sea-water that permits fertilization.

Males and females are thoroughly dried on clean filter paper. A male and a female placed in each of the eight perfectly dried clean watch glasses. Sea-water added as follows:

No. 1	1 drop.
" 2	2 drops.
" 3	3 "
" 4	4 "
" 5	5 "
" 6	6 "
" 7	10 c.c.
" 8	no sea-water.

The worms were then cut up and flooded with sea-water, later transferred to fresh sea-water in finger bowls.

Nos. 1, 2, 3 and 8 gave cleavage; a per cent. of normal trochophores was found the next morning. In dishes 4, 5, 6 and 7 not an egg divided, no swimming forms developed.

No single observation in the whole work was made as often as this; the results are wonderfully precise. As I shall show later the experiment quoted was conducted under the optimum conditions, and yet it shows the inhibiting effect of such a surprisingly small quantity of sea-water. All other observations show two drops of sea-water for each worm to be the maximum that will permit normal fertilization. In no case have I got cleavage where two and one-half drops of sea-water for each worm (*i. e.*, five drops to two worms) were used. While the same pipette was used to secure equal drops, the worms, females particularly, vary in size. I have usually taken the average females for these experiments. Such an animal, as found by actual count in three cases, has about 11,000 eggs. There is enough variation, however, in the size and weight of the worms to make impossible any law concerning the lethal amount of sea-water. I believe, nevertheless, that there is an optimum time for the addition of sea-water—equal to the time the sperm are in the female in normal insemination; and an optimum amount of sea-water—about as much as the worms will take up after thorough drying.

The results of these inseminations over a period of four seasons prove clearly that sea-water except in minute quantity is fatal to fertilization.

Does Sea-water Injure Egg, Sperm, or Both?

Three explanations of the failure of *Platynereis* eggs to cleave after insemination in sea-water are possible:

(*a*) Both eggs and spermatozoa are injured by the sea-water.

(*b*) The sperm alone are injured by the sea-water.

(*c*) The eggs alone are injured by the sea-water.

The failure of the eggs to go beyond maturation may be due to the injurious action of the sea-water on both eggs and sperm alike. It would seem reasonable to assume that for internal insemination both cells need the perivisceral fluids. It might be difficult to conceive how this adaptation in *Platynereis* could have taken place acting on one only of the sex elements. As both eggs and spermatozoa are protected by body fluids in normal insemination, so both are exposed to the lethal action of sea-water. Embryologists are all careful when inseminating eggs of forms in which insemination normally taken place in the sea not to contaminate the dishes containing ova with the animal's tissues or fluids. Lillie ('13*b*, '14) has shown why this is essential. I have, however, repeatedly with success fertilized *Nereis* eggs dry (see Just, '15*b*) doubtless because the body fluid of *Nereis* is practically negligible. And the case of *Platynereis* is similar to that of *Nereis*; in this smaller worm there is no more fluid; the female is a mere locomotor ovary, although the male does have a small amount of fluid and a great number of corpuscles.

The second possibility is that the sperm alone are injured by the sea-water. Injury to the sperm through transference from the male's body fluid to sea-water, however, cannot be due to difference in osmotic pressure. For as Frédericq has shown, and Garrey since for the Woods Hole region, the osmotic pressure of invertebrate body fluids is about the same as that of sea-water. Moreover, *Platynereis* sperm in sea-water as far as I could determine exhibit none of the effects experimentally produced by Koltzoff on various sperm cells including those of *Nereis* (*dumerilii?*) through treatment by various salt solutions or those conditions described by de Meyer with hypotonic and hypertonic solutions. In some other way, then, the sperm must be assumed to be weakened but still capable of partially fertilizing the egg as the Hertwigs, Gemmil, Budington, Dungay, etc., have shown. And indeed my *Platynereis* slides of sea-water inseminated eggs show similarities to the figures by Lillie of the penetration of injured sperm in *Nereis*; in *Platynereis*, however, the germ nuclei develop

a little farther. Steinach long ago, later Walker ('99, '11) and Hirowaki have shown that in mammals the prostate secretion is necessary for fertilization. Sea-water, then, might injure the sperm and hinder fertilization by destroying a supporting medium necessary for fertilization. (On this point, cf. Gemmil's experiments.)

Finally, a third explanation is possible: the egg alone is injured through sea-water treatment. The egg, in this case, may be dependent on a substance in the female's body or on some secretion of its own necessary for fertilization. Both egg and sperm may need body fluids but sperm may be hardier, egg less resistant.[1]

The seasons of 1912 and 1913 were largely given over to experiments to determine which possible explanation is valid for *Platynereis*. In 1914, many of these experiments were repeated. And I may say at once that the explanation must come under the third head as shown by the following experiments.

The Experiments.

The plan of the experiments is briefly as following:

Males and females were cut up separately in dishes of clean sea-water. The bits of tissue were carefully removed, the dish of eggs being handled with utmost care to prevent unnecessary agitation. The eggs and sperm suspensions were filtered after having remained in sea-water for varying lengths of time. Sexual products treated thus are designated "washed eggs" and "washed sperm."[2]

Males and females were thoroughly dried on filter paper or clean sheer linen. The males were cut up in dried clean watch glasses; the females were cut up in the same way or pricked when

[1] That the resistance of eggs and sperm of both *Nereis* and *Platynereis* is unequal would seem probable from the following: If to a *Nereis* sperm suspension janus green be added the fertilizing power of the sperm is in no wise impaired; or if the dye be added to sea-water the living males absorb it readily without any injurious effect on the sperm. The same quantities of the dye in sea-water is toxic to the egg before or at insemination. Eggs taken from a female *Platynereis* that has been swimming in a janus green-sea-water solution that is not toxic to the males or their sperm will not fertilize. Cf. also action of nicotine on *Strongylocentrotus* sperm and eggs as observed by the Hertwigs.

[2] Several methods were used for "washing" sperm and freeing them of sea-water, among others that of centrifuging at high speed for six minutes. These were all abandoned for the method here described.

most of the eggs that escaped were collected in dry watch crystals. Bits of tissue were always removed. Such eggs and sperm are "dry eggs" and "dry sperm."

For a given experiment eggs and sperm were mixed and after an interval of time varying from five to sixty seconds flooded with sea-water. Four kinds of inseminations were made:

Washed eggs × *washed sperm.*
Washed eggs × *dry sperm.*
Dry eggs × *dry sperm.*
Dry eggs × *washed sperm.*

The experiments fall into two groups: "A.M. inseminations" —made the morning after the worms were captured; and "P.M. inseminations"—made during the evening of capture.

The following table gives a summary of results:

TABLE I.

Eggs.	Sperm.	Group.	Development.
Washed	Washed	A.M. and P.M.	None.
Washed	Dry	A.M. and P.M.	None.
Dry	Dry	A.M. and P.M.	Cleavage and larvæ.
Dry	Washed	A.M.	None.
Dry	Washed	P.M.	Cleavage and larvæ.

Washed eggs, inseminated with dry or washed sperm, never reach cleavage stages nor do they ever produce swimming forms.

I have commented above on the *dry egg* × *dry sperm* series. These eggs cleave and later produce normal larvae.

Washed sperm × *dry eggs* of the A.M. group (1912) did not yield cleavage or swimming forms. The worms do not thrive well in the laboratory. The practise, therefore, of conducting experiments the morning after capture has been since 1912 practically abandoned. The only test for the vitality of the worms is copulation—a test the very nature of which precludes experiment. Doubtless, therefore, this set of experiments gave no results because the animals were not fit. Study of sections of eggs normally inseminated and laid as early as 5 A.M. shows a large percentage in the germinal vesicle stage. I have made counts in dishes of living eggs to show at the later cleavage stages the proportion of eggs still in the germinal vesicle stage. For example,

August 8, 1912, 2 P.M., six hours after laying of 10,851 eggs (from one female) six per cent. were still in the germinal vesicle stage. Other counts of living eggs and of sections show higher percentages. Every egg laid the night of capture cleaves. Dry inseminations, day or night, at best never give more than ninety per cent. of cleavages. The poor quality of the animals after several hours in the laboratory may account for the failure of the *dry eggs* × *washed sperm* A.M. group to cleave. But since the *dry eggs* × *dry sperm* A.M. series gives cleavage, I am rather inclined to believe that the method used was poor: for instance, the filter paper then used was too soft allowing the loss of most of the spermatozoa or too much water was left when the dry eggs were added.

The results with *dry eggs* × *washed sperm*, P.M. group are wonderfully uniform and show conclusively that the sea-water, at least for the exposures used, has no harmful effect on the sperm. The method used is simple. As soon as possible after capture one to three males are cut up in from 8 drops to 20 c.c. of sea-water and allowed to stand upward to twenty minutes. (The sperm are active after having been in sea-water for twelve hours.) The sperm suspension is then filtered. I used a very hard filter paper. This paper was then tilted and thoroughly drained until under the lamplight the glistening water was thoroughly absorbed. A dried female was cut up on the filter paper or pricked and the eggs thus procured rolled over the paper to reach the sperm left behind or caught in the pores of the filter. The whole was then put in a dish of clean sea-water. It would be tedious to cite the individual experiments. They show conclusively that dry eggs inseminated with washed sperm develop in normal fashion.

Now since, as has been shown above, there is a minimal amount of sea-water that will permit fertilization, dry eggs ought to fertilize if put on the filter paper before all the water has been absorbed. Such indeed is the case. Moreover, dry eggs put in two drops of thin sperm suspension develop. From a suspension made by cutting up one or more males in sea-water two drops are taken. Dry eggs put in this cleave and next morning swim.

This observation led to a series of experiments (during 1913 and 1914) designed to ascertain whether or not the density of the sperm suspension is a factor in the fertilization of *Platynereis*.

These experiments prove in general that the number of dry eggs added to sperm suspensions that develop depends upon the density of the suspension. The denser the suspension the larger the number of trochophores. Moreover, for dense suspensions the minimum amount of sea-water permitting fertilization appears to be slightly higher than for thin suspensions. Cleavage is directly a function of the chances of the spermatozoa reaching the egg before the fertilizing substance is lost.

The time of flooding with sea-water after insemination is also important for the highest percentage of cleavage. But these factors cannot be expressed with mathematical exactness. Some points, particularly with reference to inseminations with dense suspension need further experiments to determine their significance.

That the egg when exposed to the action of sea-water quickly loses something necessary for fertilization must be the conclusion drawn from these experiments with washed or unwashed eggs. Even *thirty seconds* residence in sea-water, as repeatedly proved, is sufficient to inhibit cleavage in every single egg. If dry eggs from a single female be put in five cubic centimeters of sea-water and thoroughly drained as soon as they settle they will not develop after insemination although this procedure may take but a half minute. The egg alone is affected by sea-water; the fertilizing power of the sperm is not affected by exposure to sea-water.

3. *The Nature of the Fertilizing Substance.*

The fertilizing substance once lost cannot be restored. If washed eggs be mixed with an extract obtained by crushing dry eggs in one or two drops of sea-water and dry sperm added, cleavage does not result. I lay no stress on this, however, for it seems to me that such an extract might yield anything.

The presence of various substances in the sea-water or the lowering of the temperature of the sea-water does not prevent or restore the loss of this substance.

KOH.—Eggs were teased out of the female directly into sea-water plus KOH in various proportions. Or, eggs from dried females were placed in the solution. After remaining from thirty seconds to two minutes in the alkaline sea-water the eggs were inseminated dry and flooded with sea-water. In other cases inseminations were made in the solutions. Washed eggs were similarly treated. Whatever the method alkaline sea-water never gave cleavage. (Cf. sections on cross fertilization and artificial parthenogenesis.)

Hypertonic and Hypotonic Sea-water.—Eggs, both washed and dry, were treated with 2½ M KCl + sea-water as follows:

1. 1 drop 2½ M KCl + 19 drops of sea-water.
2. 2 drops " " + 18 " " "
3. 3 " " " + 17 " " "
4. 4 " " " + 16 " " "
5. 5 " " " + 15 " " "
6. 6 " " " + 14 " " "

Dry sperm were added at once and the dishes flooded with sea-water after five minutes. Or, after treatment for varying number of minutes the eggs were inseminated dry. The eggs developed no farther than with KCl treatment alone (see beyond); they form jelly and maturate.

Hypotonic solutions used similarly gave no cleavage.

Ether.—The following table is a summary of the experiments with ether:

Eggs.	Solutions Used.	Exposure.	Inseminations.
Washed,	.3 to .6 per cent.	1 to 5 minutes dry;	in the solution.
Dry,	"	" "	" " "
Teased,	"	" "	" " "

"Teased" eggs are those got by cutting up the female in the ether-sea-water.

A few eggs form jelly and maturate after the ether treatment. Compared with sea-water inseminations, ether cuts down the per cent. of maturations. According to R. S. Lillie ('12) starfish eggs resistant to fertilization may be rendered normal by ether in low concentration. In *Platynereis* the condition is different. The egg is not rendered resistant to fertilization by the action of sea-water; it is weakened through loss of something

by the sea-water since it combines but feebly with the sperm The ether as in *Asterias* renders the *Platynereis* egg irritable since as shown by the low percentage of maturation more fertilizing substance must be secreted.

KCN.—Inseminations made with washed or dry eggs during or after treatment with KCN (1 per cent. KCN and sea-water made in various proportions) gave only maturation. But the eggs will maturate in KCN alone while in the solutions. (Cf. Allyn on *Chætopterus.*)

$CaCl_2$.—Newman found that $CaCl_2$ inhibits fertilization in *Fundulus* through a precipitation effect. I thought that in somewhat the same way calcium chloride might through action on the cortex inhibit the loss of the fertilizing substance in *Platynereis.* M/2 $CaCl_2$ added to sea-water in different quantities does not inhibit the loss of the substance since after the calcium chloride treatment the egg does not fertilize.

Cooled Sea-water.—Sea-water was cooled to 10.5° C. and dry eggs after 30, 60 and 90 seconds' treatment in 5 c.c. were inseminated at this temperature or after the cooled water was pipetted off. In some experiments the female was kept at the low temperature for several minutes before the eggs were cut out. 5 c.c. of sea-water were used in each experiment. The eggs never cleave, but more form jelly and maturate than controls inseminated in ordinary sea-water. This would seem to indicate a slowing down of the secretion. The effect of cold is just the opposite of the effect of ether. Unfortunately, only few of these experiments were made. Perhaps they should be repeated at lower temperatures.

Concerning the nature of this substance, some of my earliest notes are of interest. After insemination in sea-water I found some time later (forty minutes in one case) "sperm dancing above the eggs." In 1914, I found the sperm of sea-water insemination active after twelve hours. One does not find this after dry insemination, even with excess of sperm. Sperm in the dishes of successfully inseminated eggs are profoundly changed. Study of the movements of *Platynereis* sperm reveals the circular swimming of echinid spermatozoa, as shown by Buller, Gemmil, Winslow, and others (see also Dewitz, Ballowitz, etc.). They

finally become quiescent through lack of oxygen[1] in various positions without orientation. After dry inseminations they come to rest, as can be seen after flooding the dishes, definitely oriented and not in haphazard arrangement. Clustered among the jelly hulls, their heads point toward the eggs. On occasions, I believed that I demonstrated the agglutination of the sperm by sea-water in which the eggs had been lying. The evidence is not clear-cut and more recent attempts have failed. The egg charged sea-water, however, does activate the sperm.

I wish to point out the serious difficulties experienced in the series of sperm agglutination experiments. In the first place, twenty "large" dried males (two and one half centimeters long) do not yield enough sperm and body fluid to make up a drop as large as a drop of dry sperm from a very small *Nereis*. Then again the thickest suspension got is largely made up of blood corpuscles. I have never succeeded in procuring a "milky suspension"—the admixture of corpuscles and body fluid giving always a pinkish mixture. And finally, one cannot always get twenty or more males necessary to make up even this thin sperm suspension. Repeated efforts, therefore, extending through two seasons have not been marked with very positive results.

With *Nereis* sperm, the case is indisputable. If water in which *Platynereis* have laid eggs be taken it is found to have an agglutinating effect on *Nereis* sperm. Thus:

August 18, 1914. At 10:15 P.M., ten females laid eggs in six c.c. of sea-water each. After five minutes some of this water was drawn off—20 c.c. in all. *Nereis* sperm suspensions were made up fresh at 10:20, 10:30, 11:00 and 11:05. A drop of the sperm suspension was mounted on a slide under a raised cover slip. A drop of the water taken from the dishes of eggs was injected beneath the cover slip. Under the microscope, the quiescent sperm appeared at first intensely active, then rushed together and formed agglutinated masses among others still free-swimming.

[1] This fact was brought out in 1913 when I was repeating some old observations on echinoderm spermatozoa. While experimenting with the sperm of *Thyone* in janus green solutions, I noted after some time had elapsed that cover-slip preparations showed that bacteria present previously bluish in color had changed to a decided red. Later observations proved that as the dye was reduced in bits of tissue under the cover slip the sperm quieted down in various positions.

The same experiment succeeds if one uses the water from dishes in which uninseminated eggs have remained for a few minutes. Washed eggs do not cause agglutination of *Nereis* sperm; water charged by normally inseminated eggs or uninseminated eggs retains its power of agglutinating *Nereis* sperm after twelve hours at least, the reaction coming on more slowly. The freshly charged water acting on fresh sperm suspension gives a clear-cut and beautiful reaction.

It may seem far-fetched to argue that the fertilizing substance lost by *Platynereis* eggs when exposed to sea-water is agglutinin or fertilizin as discovered by Lillie in *Nereis* and *Arbacia* because the washed egg, no longer fertilizable by its own sperm, can not sufficiently charge the sea-water to agglutinate *Nereis* sperm. Yet I believe this is the case precisely. The agglutination of *Nereis* sperm by *Platynereis* egg-water is correlated with jelly formation in *Platynereis* by *Nereis* sperm. In sea-water inseminations, *Nereis* spermatozoa are almost as effective as those of *Platynereis*. Added to this is the difference in behavior of *Platynereis* sperm in egg charged sea-water, in sea-water inseminations, and in dry inseminations.

The evidence may be scant, but it seems to me sufficient to indicate that the substance lost which is necessary for fertilization is identical in nature with the fertilizin of Lillie.

B. Cross Fertilization with Nereis.

I have mentioned (Just, '14) the fact that it is generally taken for granted that reciprocal crossing of *Nereis* and *Platynereis* is the rule. This led me to attempt cross fertilization. Cross fertilization never produces segmentation or development though it may induce the maturation process.

Of the methods used in echinoderm hybridization—those of Loeb, Tennent,[1] etc.: (1) high temperature; (2) treatment with fresh water; (3) treatment with alkalis; (4) allowing the eggs to stand; and (5) polyspermy—all were tried except the first. Since the eggs of *Platynereis* are normally inseminated in the body cavity and therefore with little sea-water, I tried "dry

[1] Dr. Tennent in 1912 very kindly communicated to me at length his latest methods in echinoderm hybridization.

inseminations": *i. e.*, *Nereis* males were cut up dry and a drop of the sperm without the addition of sea-water added to eggs of *Platynereis* cut up dry. Inseminations were made in a variety of ways as the following table of method shows:

TABLE II.

SUMMARY OF INSEMINATIONS MADE IN 1911, 1912, 1913, AND 1914

Platynereis sperm	on	*Nereis* egg.
1. Few sperm in sea-water. 2. Dense sperm suspension.		Fresh eggs in sea-water.
3. Few sperm in sea-water. 4. Dense sperm suspension.		Stale eggs in sea-water.
5. Few sperm, dry, 6. Heavy insemination dry.		Fresh eggs dry.
7. Few sperm, dry. 8. Heavy insemination, dry.		Stale eggs washed.

Reciprocal crosses of *Platynereis* eggs and *Nereis* sperm were made.

"Stale eggs" are eggs that have stood in sea-water for several hours. "Stale eggs, washed" are stale eggs on which the water has been changed several times.

These experiments were made repeatedly during four seasons. The sperm of *Platynereis* has practically no effect on the egg of *Nereis* whether fresh or stale, dry or in sea-water. In one experiment (1911) I got jelly formation in a few eggs. This experiment later repeated (1913) gave no result. If *Nereis* eggs be inseminated with *Platynereis* sperm during the evening of capture they show no change the next morning. Inseminated with *Nereis* sperm twelve hours after insemination with *Platynereis* sperm, the eggs develop normally if anything in greater numbers than such stale eggs in ordinary sea-water do.

Nereis sperm will cause *Platynereis* eggs to form jelly, the per cent. of eggs thus responding depending upon the amount of sea-water used and the density of the sperm suspension. But in general many of the eggs fail to form jelly or go through maturation. Many that maturate do so with the cortex partially or wholly intact. Sections of these eggs preserved at three minute

intervals after insemination have been studied. The sperm does not enter; or, if it enters must disintegrate early for I have never found sperm nuclei in these preparations.

Clearly, then, one may not use the eggs of these worms indiscriminately.

C. Artificial Parthenogenesis.

The following agents have been used in an attempt to bring about artificial parthenogenesis in the egg of *Platynereis megalops*:

1. Centrifuging,
2. KCl,
3. NaOH,
4. KOH,
5. HNO_3,
6. HCl,
7. Warm sea-water.

The eggs were cut out of the worms in sea-water centrifuged; subjected to varying quantities of salt, alkalis, or acids for different lengths of time; or warmed in sea-water for from five to thirty minutes at 35° C. These methods gave polar body formation, cytoplasmic changes, fusion of the oil drops, and finally chromatin disintegration in the animal hemisphere. The eggs never cleaved.

Study of the literature reveals the fact that the clearest cases of artificial parthenogenesis closely simulating the normal in cleavage and in larval development are of those eggs that have formed one or both polar bodies when shed: the echinids, for example, and the asteroids. Other eggs shed in the germinal vesicle stage like those of *Polynoe* (Loeb '08), *Amphitrite* (Loeb '01; Scott.) *Nereis* (Lillie '11), etc., give only differentiation without cleavage or incomplete cleavage. Loeb and Wasteneys' work on *Chaetopterus* with ox serum as well as Miss Allyn's on the same egg with heat are exceptions. The great exception to the general statement made above is *Thalasema* (Lefevre) where it appears with single substances, acids mostly, normal development is closely simulated. On the whole, however, ovocytes yield less readily to parthenogenetic agents than mature ova.

Mathews' experiments ('01) on *Asterias* may in this connection be cited. He found that when the eggs of this starfish were got while still in the germinal vesicle stage shaking would produce development only after the eggs had remained in sea-water until maturation was gone through with. Sea-water acts as a first stimulus and mechanical shock induces further development. So R. S. Lillie ('08) on the same egg finds that its responsiveness to momentary elevation of temperature as a means of producing artificial parthenogenesis "varies greatly at different periods in the life of the egg." "The most favorable period is some little time (10 to 20 minutes) before the separation of the first polar body."

Reasoning thus, I thought that I might carry *Platynereis* eggs through maturation with one agent and then through cleavage with another. Eggs were, therefore, treated with KCl, KOH, and NaOH in sea-water for various lengths of time and then subjected to heat, shaking, and centrifugal force. In no case did I procure cleavage although the first agent in each case caused maturation. With *Nereis*, on the other hand, KCl and subsequent warming in sea-water induces development (see Just '15*b*).

It is interesting to note that eggs subjected to heat in the minute quantities of sea-water that permit fertilization do not develop beyond maturation. Apparently, the conditions for successful artificial initiation of development are more exacting than those for successful insemination.

We may conclude, then, that the results of attempted cross fertilization and artificial parthenogenesis are harmonious with those of sea-water insemination, so far as cleavage is concerned, in their negative results. The fundamental questions are: (1) the significance of the sea-water insemination and (2) the extent to which the results with *Nereis* sperm and with parthenogenetic agents are capable of like interpretation.

Discussion.

Any analysis of fertilization must deal with the phenomena from the point of view of heredity or of initiation of development. Considered as the process of initiating development, fertilization may be divided into the stages of insemination, sperm penetration, and germ nuclei copulation. As Lillie has repeatedly

pointed out[1] experimental evidence must be amassed testing the meaning of each of these stages.

1. Concerning insemination, as Lillie has shown, the egg plays an important part through the production of agglutinins.[2] For both *Arbacia* and *Nereis* it has also been shown that chemotaxis plays a part in insemination. (Lillie, '12, '13*a*, '13*b*, and '14).

I believe that *Platynereis* belongs to this class. I may, however, be permitted again to point out the great difficulty attending the use of *Platynereis* eggs on this phase. All the phenomena are extremely rapid, the reactions must be very nice. The material is unfavorable for any intensive study of agglutination and chemotaxis. When one stops to think of the extremely precise reactions of the eggs, one gets a hint of the task. The carrying over of the *smallest* drop of sea-water above the maximum to eggs from vigorous females within the shortest time after capture will prohibit cleavage in every egg.

To answer the general question whether or not eggs secrete substances that activate the spermatozoa, I believe forms whose eggs are inseminated normally in sea-water should be used. So far as *Platynereis* is concerned, agglutination or not, chemotaxis or not, the egg must lose a substance or substances when in sea-water whose presence is necessary for fertilization.

2. Study of the normal fertilization of *Platynereis* indicates that as in *Nereis* the egg plays the active rôle in the penetration of the spermatozoön for it actually draws in the passive spermatozoön. After sea-water treatment I have not, as mentioned above, found the early stages of penetration in eggs fixed at three minute intervals after insemination. Either the sperm penetration is unlike that after normal insemination or penetration takes place with extreme rapidity. In the later stages of penetration it is

[1] Lectures to classes in embryology, Woods Hole, Mass.

[2] Apparently Buller did not realize that he obtained iso-agglutination of sea-urchin sperm, although he speaks of the sperm forming "balls" and although the phenomena of agglutination were well known at that time. Landsteiner the year before had secured sperm agglutinating sera. Nougouchi's work on *Nereis* sperm is of interest: he demonstrated agglutination with snake venom. The experiments of Schücking, von Dungern, de Meyer, and others are well known. An observation of Walker's ('10) is likewise worthy of mention—the agglutination of the sperm of the rat when mixed with the seminal vesicle secretion of the same animal.

Chemotaxis of sperm has been demonstrated for mammals—see for instance, Löw.

clear that the spermatozoa behave in abnormal fashion even granting that I may have overlooked the amphiaster. The evidence seems to indicate that after sea-water treatment the egg lacks the power to engulf the sperm. However, whatever the method of penetration one point is beyond contradiction: these washed eggs never cleave.

The observations agree with those of Lillie ('14) who notes that some unpublished observations in the case of *Nereis* show that "if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoön, but without causing cleavage of the egg." Miss Allyn found that after KCl treatment of the egg of *Chætopterus*, the spermatozoön may enter but its behavior is not normal. Kite (quoted from Lillie '14) finds that spermatozoa injected into star-fish eggs never give cleavage.

In these cases, the interpretation must be that the "fertilizable" condition of the egg has been destroyed through loss of fertilizin before insemination. In the same way sperm may penetrate unripe eggs as Hempelmann has shown for *Saccocirrus* (so too, von Hofsten for *Otomesostoma* and Shearer for *Dinophilus gyrociliatus*). Two years ago I found that eggs from *Nereis limbata* just before transformation into the heteronereis phase would not fertilize with active sperm either from the nereis or heteroneris form. Moreover, eggs from metamorphosing worms kept for several weeks in the laboratory although apparently ripe would not fertilize on insemination during the dark of the moon. At full moon, sometimes but a few days later, eggs from the same animal would fertilize and develop into larvæ which were kept for weeks. We may assume in these cases that the fertilizin is either absent or is unavailable. Penetration, therefore, may take place before the fertilizable period is reached as well as after it has been passed, but the egg is not capable of fertilization.

3. Apposition of the germ nuclei of *Platynereis* after sea-water insemination may ensue, but never cleavage. After the loss of the fertilizing substance, then, the normal fertilization process may be closely simulated even to the point of the copulation of the pronuclei but development never goes beyond this point. In short, the normal fertilization process demands at the very

outset the fixation by the spermatozoön of the escaping fertilizin. This takes place in *Platynereis* almost instantaneously (see page 93) but brief though this phase may be it cannot be omitted.

The experiments with *Nereis* sperm and agents of artificial parthenogenesis demand explanation. Eggs such as those of echinids used in cross fertilization (Loeb, Tennent, Baltzer, Herbst, etc.) or in artificial parthenogenesis when subjected to treatment are so subjected with their substances intact. They are normally shed in sea-water for insemination and the sea-water does not for some time destroy their fertilizing power. *Platynereis* eggs when subjected in sea-water to foreign sperm or to various agents have lost something through the action of sea-water. This very "something" is necessary for artificial parthenogenesis and, moreover, as shown above (for *Nereis* also) must be present in greater quantity than necessary for fertilization. I am emboldened further to suggest that eggs normally inseminated in the ovocyte stage yield to parthenogenetic agents only with difficulty because they lose fertilizin at the impact of the first stimulus—chemical treatment, shock, etc. Sperm alone, in most cases, are strong enough by fixation of the fertilizin to carry such eggs through their dual phase—maturation and fertilization. Whether by sperm, then, or by artificial agents, the initiation of development is fundamentally the same.[1] The egg plays the leading rôle; it needs but to have its fertilizin activated in order to develop.

The observations on *Platynereis* were rendered less difficult because of the study of the maturation and fertilization in *Nereis*. For this study I was fortunate to be able to supplement my own slides with two series lent me by Professor F. R. Lillie. It is a genuine pleasure here to acknowledge my further indebtedness to him for his many suggestions and for his stimulating interest in the *Platynereis* studies begun at his suggestion and under his direction.

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[1] I think that Martin Jacoby's experiments support this view. He found (*Biochem. Zeit., 26, 333–335*) that serum from rabbits into which eggs had been injected showed an increased power to stimulate parthenogenetic development of the eggs. He also found (*ibid., pp. 336–343*) that an enzyme which may be extracted from sperm and from eggs after sperm penetration may be got from parthenogenetic eggs.

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